
A comparison of non-radioisotopic hybridization assay methods using fluorescent, chemiluminescent and enzyme labeled synthetic oligodeoxyribonucleotide probes

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ABSTRACT

N⁴-[N-(6-trifluoroacetylamidocaproyl)-2-aminoethyl]-5'-O-dimethoxytrityl-5-methyl-2'-deoxycytidine-3'-N,N-diisopropyl-methylphosphoramidite has been synthesized. This N⁴-alkylamino deoxycytidine derivative has been incorporated into oligonucleotide probes during chemical DNA synthesis. Subsequent to deprotection and purification, fluorescent (fluorescein, Texas Red and rhodamine), chemiluminescent (isoluminol), and enzyme (horseradish peroxidase, alkaline phosphatase) labels have been specifically incorporated. Detection limits of the labels and labeled probes were assessed. Also, the detection limits and non-specific binding of the labeled probes in sandwich hybridization assays were determined. The enzyme modified oligonucleotides were found to be significantly better labeling materials than the fluorescent or chemiluminescent derivatives, providing sensitivities comparable to ³²P-labeled probes.

INTRODUCTION

Radiolabeled polynucleotides probes have been extensively employed for the detection of complementary nucleic acids by specific hybridization (1). Within the last few years considerable attention has been given to methods for incorporating non-radioisotopic labels into polynucleotides in order to circumvent the problems inherent to radioactivity. Also, some non-radioactive labeling systems may lead to significantly improved detection limits.

The most important criteria in developing a non-radioactive labeling scheme are maximization of the label density and minimization of non-specific binding (NSB) of the labeled probe. With large polynucleotides (over 100 bases), it is difficult to separate partially or unlabeled material from the fully modified species regardless of the label employed. As a result, typically no purification is attempted after labeling so that the label

density is dictated only by the yield of the coupling reaction (2,3). However, with short synthetic oligonucleotides the resolution provided by PAGE, HPLC or column chromatography is sufficient to permit separation of fully modified probes from all other reaction components (4-9). The number and position of labeling moieties per polymer can be adjusted to whatever is desired, thus determining the label density.

Small labeled oligonucleotide hybridization probes offer additional advantages over larger polynucleotides. Large quantities (10-100 nanomoles) can be inexpensively produced by chemical synthesis. Since the change in T_m as a function of length is more dramatic with polynucleotides less than 20 bases (10), it is relatively simple to favor the formation of the intended hybrid over mismatches (11).

Most non-radioactive labels are difficult to incorporate directly during the chemical synthesis of oligonucleotides since they are not stable in the reagents employed for synthesis and/or deprotection. As a result, appropriately blocked nucleophilic "handles" such as alkyl-sulfhydryls (12-14) or -amines (4-9) have been incorporated during the solid supported synthesis of DNA. Subsequent to deprotection and purification of the probes, these sites can be used to direct the introduction of nucleophile-specific labeling reagents.

We report here the synthesis of a fully protected 3'-phosphoramidite of an alkylamine derivative of deoxycytidine and its use in the synthesis of oligodeoxyribonucleotides. These alkylamine-containing oligonucleotides have been modified with fluorescent, chemiluminescent and enzyme moieties. Biotinylated oligomers have also been produced and shown to be useful as capture probes in sandwich type nucleic acid analysis. Application of the labeled probes in an assay for a simple DNA analyte suggests that horseradish peroxidase (HRP) and alkaline phosphatase (AP) are superior to the fluorescent and chemiluminescent reporter groups tested.

MATERIALS AND METHODS

Synthesis of the N⁴-alkylamino deoxycytidine 3'-phosphoramidite

4-Triazolopyrimidinone nucleoside was synthesized from 5'-O-dimethoxytrityl thymidine as described by Reese et al. (15),

using transient 3'-O-trimethylsilyl protection (16). TLC was performed on silica gel 60 F254 plates in 10% methanol/CH₂Cl₂. Twenty five grams (46 mmoles) of 5'-O-dimethoxytrityl thymidine ($R_f = 0.63$) was dissolved in 150 ml of dry CH₃CN in a 1 L round bottom flask. After the addition of 50 ml of N,N-dimethylamino-trimethylsilane (Petrarch Systems), the solution was stirred at room temperature for 30 min. The mixture was then evaporated to dryness on a rotary evaporator to give 5'-O-dimethoxytrityl-3'-O-trimethylsilyl-thymidine (compound 1) in quantitative yield ($R_f = 0.70$). 1,2,4-Triazole (51.2 g) was dissolved in 300 ml of CH₃CN and 16 ml of POCl₃ was added. The solution was set on ice and upon the dropwise addition of 120 ml of triethylamine, the mixture became a thick slurry. The material was diluted by the addition of 100 ml of CH₃CN. The oily residue of compound 1 was dissolved in 100 ml of CH₃CN and added dropwise to the reaction flask. Stirring was continued for 60 min on ice, then 30 min at room temperature. The solution was diluted with 800 ml of ethyl acetate and extracted twice with each 800 ml of 5% aqueous NaHCO₃ and 800 ml of 80% saturated aqueous NaCl. After drying the organic phase over Na₂SO₄, the solvent was removed by evaporation under vacuum at room temperature. Upon co-evaporation with toluene then CH₃CN, 31 g of 4-(1,2,4-triazolo)-1-(β -D-5-O-dimethoxytrityl-3-O-trimethylsilyl-2-deoxyribofuranosyl)-5-methyl-2(1H)-pyrimidinone (compound 2) was obtained and used without further purification ($R_f = 0.65$; fluorescent blue spot under UV examination).

The 1,2,4-triazole moiety was displaced with ethylene diamine as reported by Sung (17) and Maggio et al. (18). Compound 2 was dissolved in 200 ml of CH₃CN and added to a solution containing 25 ml of ethylene diamine in 200 ml of CH₃CN on ice. After 15 min, the reaction mixture was extracted and dried as above to yield 26.8 g (40.6 mmoles) of 5'-O-dimethoxytrityl-3'-O-trimethylsilyl-N⁴-(2-aminoethyl)-5-methyl-2'-deoxycytidine (compound 3) (ninhydrin positive spot at baseline by TLC).

Compound 3, in 200 ml of CH₂Cl₂, was reacted with 100 ml of a 0.5 M solution of N-hydroxysuccinimidyl N-trifluoroacetyl-6-aminocaproate, prepared essentially as described elsewhere (19), at room temperature for 30 min. Subsequent to evaporation of the material to dryness and co-evaporation with 250 ml of toluene,

the resulting foam was dissolved in 400 ml of anhydrous methanol. In order to remove the 3'-O-silyl protection, 55 ml of 1 M K₂CO₃ was added and the reaction was stirred for 15 min at room temperature. At this time, the solution volume was reduced to approximately 500 ml by rotary evaporation. After the addition of 700 ml of ethyl acetate, the mixture was extracted and dried as above to give N⁴-[N-(6-trifluoroacetylamidocaproyl)-2-aminoethyl]-5'-O-dimethoxytrityl-5-methyl-2'-deoxycytidine (compound 4). One half of the crude sample was loaded onto a 1000 ml silica gel 60H column in 100 ml of 0.5% triethylamine in CH₂Cl₂. The column was eluted with 1200 ml, 1200 ml and 1800 ml each of 2%, 4% and 6% methanol in 0.5% triethylamine, CH₂Cl₂, respectively. The 150 ml fractions were monitored by TLC (R_f = 0.37, ninhydrin negative; prior exposure of the plate to ammonia vapor results in a positive ninhydrin test). Fractions containing compound 4 were combined and the nucleoside was precipitated from cold hexanes to yield (from two columns) 21 g (26.4 mmoles; 57% overall yield). ¹H-NMR (CDCl₃) δ 0.95 (t, 2H), 1.3 (m, 4H), 1.45 (s, C-5 CH₃), 1.5-1.6 (m, 6H), 3.2 (m, 2H). UV spectrum (ethanol), λ_{max1} = 246 nm (Σε₂₄₆ = 4900), λ_{max2} = 280 nm (Σε₂₈₀ = 5800).

Compound 4 was converted to the corresponding 3'-phosphoramidite using standard procedures (20) to yield N⁴-[N-(6-trifluoroacetylamidocaproyl)-2-aminoethyl]-5'-O-dimethoxytrityl-5-methyl-2'-deoxycytidine-3'-N,N-diisopropylmethyl-phosphoramidite (compound 5; Figure 1). ¹⁹F-NMR δ -12.5 (relative to CCl₃F). ³¹P-NMR δ 145.4 and 146.0 (relative to (CH₃O)₃PO). 2-Cyanoethyl phosphorus protection has also been utilized. Although the derivative reported here is a 5-methyl deoxycytidine (synthesized from thymidine), we have also produced a deoxycytidine analog (8; synthesized from deoxyuridine).

Oligonucleotide synthesis

All oligodeoxyribonucleotides were synthesized by a solid supported phosphoramidite chemistry (20) on the "home-made" Gene-O-Matic automated DNA synthesizer as described elsewhere (21). The typical thiophenol (for methyl phosphoramidites) and ammonia deprotections were employed (22). Oligomers were purified by PAGE under denaturing conditions (23). Following desalting on C-18 Sep-Pak cartridges (Waters) (24), the oligonucleotides were

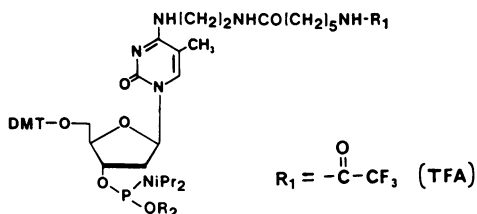


Fig.1. Structure of the fully protected N⁴-alkylamino 5-methyl deoxycytidine analog 3'-phosphoramidite. R₂ = methyl or 2-cyanoethyl.

further purified on Sephadex G-25 columns (disposable PD-10 Columns from Pharmacia) equilibrated with water. The concentration of oligonucleotides was determined by UV absorption assuming 1 OD₂₆₀ unit/ 35 µg. All derivatized probe concentrations were determined from the combined label and oligonucleotide absorbance at 260 nm.

All fragments used in the labeling studies were 5'-alkylamine derivatives, 18 or 20 bases long with a G:C/A:T ratio of 1.0 to 1.5. Although not shown here, the methods reported below have also been successfully employed to label oligonucleotides substituted with alkylamino deoxycytidine residues at the 3' end and at internal sites.

Biotin derivatization

Oligonucleotides (3-5 OD₂₆₀ units) were taken up in 100 µl of 0.1 M sodium phosphate, pH 7.5, to which 100 µl of DMF containing 1 mg of "long chain" N-hydroxysuccinimidyl biotin (Pierce Chemicals) was added. After 18 h at room temperature, the mixture was partially purified by Sephadex G-25, as above, and evaporated to dryness. The pellet was diluted with 30 µl of 90% formamide, 0.1% bromophenol blue. The material was loaded onto a 20% polyacrylamide gel (20 x 40 x 0.15 cm) and electrophoresed overnight at 7 ma. Bands were cut out, eluted and desalted as described (23,24).

Capture probe beads

Biotinylated probe (1 nanomole in 66.7 µl of water) was combined with 5 ml of a 0.25% solution (w/v) of 0.8 µm avidin beads (Pandex Laboratories, Mundelein, IL), 1 ml of 20x SSC, 0.5 ml of 1% NP-40 and 0.6 ml of 1 mg/ml poly-A. After 1 h at 37°C,

the beads were washed twice by centrifugation with 4x SSC, 0.1% NP-40 then stored in 2.5 ml of this solution until used.

Microtiter dish wells

Alkylamino probes were covalently bound to passively adsorbed proteins on microtiter dish wells (Immulon II Removawell strips; Dynatech Laboratories, Inc.) as will be described elsewhere (J.A. Running, et al., manuscript in preparation). Coatings were performed in 20 μ l.

Fluorescent probes (fluorescein, Texas Red and rhodamine)

For fluorescein derivatization, 2 OD₂₆₀ units of the appropriate oligonucleotide was dissolved in 100 μ l of 0.1 M sodium borate, pH 9, containing 2 mg of fluorescein-5-isothiocyanate (Molecular Probes Inc.) and set overnight in the dark. For Texas Red and rhodamine incorporation, 100 μ l of 0.1 M sodium phosphate, pH 7.5, was used to dissolve 2 OD₂₆₀ units of the oligonucleotide and 3 mg of Texas Red (sulforhodamine 101 sulfonyl chloride; Molecular Probes) or 5 mg of 5-(and 6-) carboxytetramethylrhodamine succinimide ester (Molecular Probes) in 100 μ l of DMF was added. The solutions were vortexed and the reactions were incubated overnight in the dark.

All reaction mixtures were then passed through a 10 ml Sephadex G-25 column previously equilibrated with 30 ml of 10 mM triethylamine acetate, pH 7.3. The colored void volume of the column was concentrated to 50 μ l by multiple extractions with *n*-butanol and dried for 20 min in a Speed Vac concentrator (Savant Instruments). The fluorescent bands (as determined by a hand held UV lamp) were cut, eluted and desalted with a C-18 Sep-Pak cartridge. The probes were stored at 4°C.

Chemiluminescent probe (isoluminol)

ABEI-H [N-(4-aminobutyl)-N-ethyl isoluminol hemisuccinimide; LKB Inc.] was converted to the N-hydroxysuccinimide ester as follows. To 50 mg (133 μ moles) of ABEI-H, 800 μ l of a 0.2 M solution of N-hydroxysuccinimide (160 μ mole) and 1.33 ml of a 0.2 M solution of dicyclohexylcarbodiimide (266 μ mole) were added and thoroughly mixed. After 18 h at 20°C, the solution was centrifuged and the supernatant was removed and evaporated to dryness. The residue was washed twice with diethyl ether then suspended in 100 μ l of DMF. This material, ABEI-HSE [N-(4-

aminobutyl)-N-ethyl isoluminol hemisuccinimide, succinimide ester], was stored at -20°C until used.

The isoluminol probe was prepared with 5 mg of ABEI-HSE in 50 μ l of DMF, then purified as described above for the Texas Red and rhodamine derivatizations.

HRP derivatization

To 10 OD₂₆₀ units of the appropriate alkylamino oligonucleotide dried in a 1.5 ml Eppendorf tube, 25 μ l of 0.1 M sodium borate, pH 9.3, and 500 μ l of distilled DMF containing 20 mg of p-phenylene diisothiocyanate (DITC; Pierce Chemicals or Aldrich) were added. The solution was vortexed and incubated in the dark at room temperature for 2 h. After transferring the mixture to a 10 ml centrifuge tube, 3 ml of n-butanol was added. The reaction was vortexed, 3 ml of water was added and the tube was vortexed again. The mixture was centrifuged and the yellowish upper layer was discarded. The extraction process was repeated with further n-butanol additions until a final volume of approximately 50 μ l was obtained. Remaining butanol was removed by evacuation, then 10 mg of HRP (Boehringer Mannheim) in 200 μ l of 0.1 M sodium borate, pH 9.3, was added. The mixture was vortexed and left overnight at room temperature in the dark.

The HRP-DNA conjugate was separated from free enzyme and oligonucleotide on a 7% polyacrylamide gel. The gel was run under standard conditions until the bromophenol blue was about 2/3 down the gel. The orange-brown bands near the middle of the gel were cut out with a razor blade and put into a 10 ml polypropylene Econo-column (Bio-Rad) to which 3 ml of 0.1 M sodium phosphate, pH 7.5, was added. The conjugate was eluted overnight at room temperature in the dark.

The contents were filtered through the frit at the bottom of the column into a Centricon 10 Microconcentrator (Amicon) prewashed twice with distilled water. The HRP-DNA was then concentrated by centrifugation at 3500 RPM and washed twice with 1 ml portions of 1x PBS. The final product was stored at -20°C.

AP derivatization

Calf intestinal AP (3 mg in buffer; immunoassay grade, Boehringer-Mannheim) was placed in a Centricon 30 Microconcentrator. Approximately 2 ml of 0.1 M sodium borate, pH

9.5, was then added and the device was spun at 3500 RPM until a final volume of 40 μ l was obtained. The alkylamino oligonucleotide to be derivatized was then activated with DITC, extracted with butanol and combined with the protein as described above. PAGE, elution (with 0.1 M Tris, pH 7.5, 0.1 M NaCl, 10 mM $MgCl_2$, 0.1 mM $ZnCl_2$), and concentration as described for the HRP conjugates were employed. The final product was stored at 4°C.

Preparation of ^{32}P -labeled probes

Fragments (50 pmoles in H_2O) to be labeled were dried by evacuation in 1.5 ml Eppendorf tubes. The probes were resuspended in 15 μ l of labeling solution containing 50 mM Tris, pH 9.5, 10 mM $MgCl_2$, 5 mM DTT, 2 units of T_4 polynucleotide kinase (Pharmacia), 100 pmoles of "crude" γ - ^{32}P ATP (New England Nuclear), then incubated at 37°C for 90 min. To precipitate the probe, 35 μ l of TE (10 mM Tris, pH 7.5, 1 mM EDTA), 2 μ l of 1 mg/ml poly-A, 25 μ l of 6 M ammonium acetate and 200 μ l of ethanol were added. After 1 h at room temperature, the samples were spun at 4°C for 10 min in an Eppendorf centrifuge. The pellets were washed with 80% ethanol and redissolved in 100 μ l of TE. The probes were reprecipitated with 10 μ l of 3 M sodium acetate and 250 μ l of ethanol at -80°C for 30 min, collected and washed as above, then resuspended in 100 μ l of H_2O containing 5 μ g of poly-A. PAGE analysis revealed better than 99% removal of unincorporated label and over 90% recovery of the probes. Cerenkov counting (1 min) was used for all ^{32}P detections using an LKB Model 1209 Rackbeta Scintillation Counter. Typically, the specific activity ranged between 1000 and 4000 cpm/femtomole of probe.

Sandwich hybridization assay procedure

Assays were conducted either with 10 μ l of beads (10 pmoles of bound capture probe) in 1.5 ml Eppendorf tubes or in a microtiter dish well (2 pmoles of bound capture probe) as depicted in Figure 2. Beads were washed by centrifugation and decantation, whereas wells were washed by aspiration.

A stock solution of the target fragment [40 bases; 10 attomoles to 10 pmoles per 20 μ l of Hyb Mix (0.1 % SDS, 4xSSC, 1 mg/ml sonicated salmon sperm DNA and poly-A, 10 mg/ml BSA)] was prepared just prior to hybridization. Triplicate samples at each

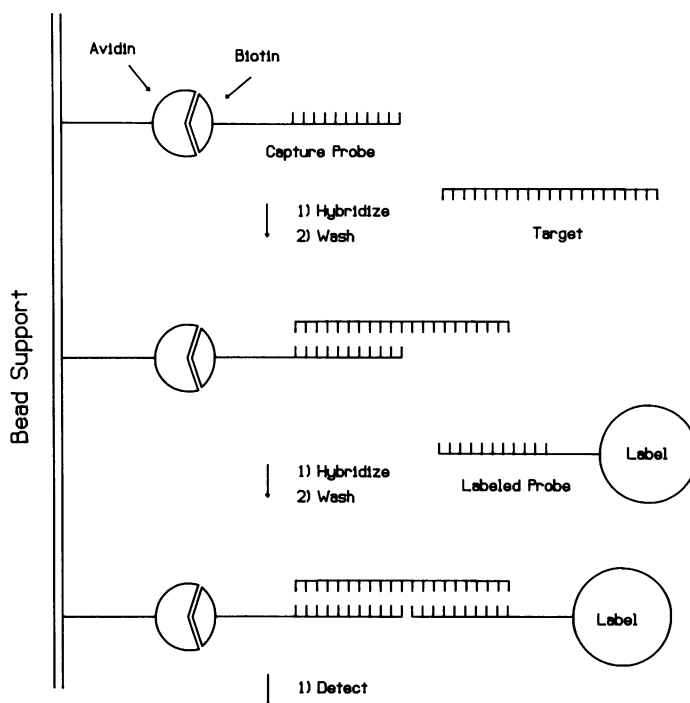


Fig. 2. Two-stage sandwich nucleic acid hybridization assay scheme.

target concentration were employed. After adding 20 μ l of target fragment solution to the beads or wells, hybridization was carried out at 55°C in a water bath for 1 h. Tubes were capped and wells were sealed with an adhesive Linbro/Titertek membrane. After washing three times at room temperature with either 0.1% SDS (or 0.1% NP40), 4x SSC for beads or 0.1% SDS, 0.1x SSC for wells, the labeled probe in 20 μ l of Hyb Mix was added in a 5-fold molar excess over the maximum target fragment concentration. The solid phase was washed three times as above, then subjected to the required detection solution as described below. A "no target" control (Hyb Mix only) was run in triplicate with each series.

Fluorescent detection

For solution emission spectra of the fluorescent dyes and probes, a Perkin-Elmer MPF-66 spectrofluorimeter was employed.

Excitation and emission wavelengths of 495 nm and 519 nm, 586 nm and 603 nm, 550 nm and 595 nm were used for fluorescein, Texas Red and rhodamine and their derivatives, respectively. These optimal excitation and emission wavelengths were determined from corrected spectra using the Perkin-Elmer survey scan software. All spectra were acquired in 1 cm cuvettes with slits width of 5 nm.

Fluorescent sandwich hybridization assay data was obtained on a Pandex Screen Machine (Pandex Inc., Mundelein, IL) by a modification of the particle concentration fluorescence immunoassay procedure (25). After conducting the hybridizations as described above, the beads were transferred to a 0.2 μ m cellulose acetate Pandex microfiltration plate and washed four times with 4x SSC, 0.1% NP-40, then dried thoroughly by vacuum. Fluorescein, rhodamine and Texas Red labels were read according to the manufacturer's suggested settings.

Detection limits for the spectrofluorimeter and Screen Machine were correlated by binding a known quantity of the appropriate dye to 0.8 μ m alkylamino polystyrene beads (Pandex). Typically, the Screen Machine was ten times more sensitive.

Chemiluminescent detection of isoluminol

Luminescence of ABEI-H, the isoluminol modified probe, and the dried beads or wells was determined with a hematin catalyst (26) as follows. To the sample, 130 μ l of 50 mM NaOH was added and each sample was mixed thoroughly. Subsequently, 20 μ l of 0.5 μ M hematin (Sigma Chemicals) in 50 mM NaOH was aliquoted into the tubes or wells. After 10 min at room temperature in the dark, 50 μ l of 90 mM H_2O_2 was added to each sample just prior to reading on a Turner TD-20e luminometer (Turner Instruments, Mountain View, CA; integration, 15 sec; smoothing, 3). Output was given as the full integral of the light produced during the reaction.

Colorimetric detection of HRP

To each vessel, a 100 μ l aliquot of a fresh o-phenylenediamine solution (OPD; in tablet form from Sigma Chemicals; 50 mg dissolved in 5 ml of 50 mM sodium citrate, pH 5.1, containing 3 μ l of 30% H_2O_2) was added. After 20 min at 37°C, 50 μ l of 4 N H_2SO_4 was added to quench the reaction. For bead assays, the beads were then pelleted by centrifugation and

the supernatant was transferred to a microtiter dish well. The dish was read on a Biotek EL310 plate reader set at 490 nm. Longer incubations did not improve the signal (S) to noise (N) ratios (S/N).

Chemiluminescent detection of HRP

A modification of the enhanced chemiluminescence (ENH/LUM) method (27,28; luminol with p-hydroxycinnamic acid) was employed (9). Luminol (LUM) alone was also employed as a substrate using the same solution without enhancer (no p-hydroxycinnamic acid). For both methods, beads were taken up in 15 μ l of chemiluminescent substrate solution, then transferred to 8x50 mm Evergreen polypropylene tubes containing 5 μ l of H₂O₂. Microtiter dish wells were treated similarly. After 30 sec, tubes were read on the Turner TD-20e luminometer (delay, 10 sec; integration, 20 sec; smoothing, 20).

Colorimetric detection of AP

A p-nitrophenyl phosphate (NPP) based detection kit (Sigma Diagnostics) was used according to the manufacturer's instructions (1.5 M 2-amino-2-methyl-1-propanol, pH 10.5). Incubations were conducted at room temperature for 1 h and samples were read at 405 nm.

The Blue Gene detection kit (BRL), which employs the nitro blue tetrazolium, 5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP) system, was also tested. Incubations were carried out until the blue precipitate was visible to the naked eye (4-18 h).

RESULTS

Although we have investigated numerous techniques for the purification of non-radioisotopically labeled oligonucleotides, we have found PAGE to be the most convenient and reliable method. Figure 3 shows the electrophoretic mobility profile of several different labeled oligonucleotides. In each case, all reaction components were well separated. Since the fluorescent and chemiluminescent labeling reagents caused significant sample smearing during PAGE, the reaction mixtures were first passed through a molecular sieving column. Typically, nanomole quantities of probe were modified and purified. Since it is usual that femtomoles of labeled probe are required for hybridization

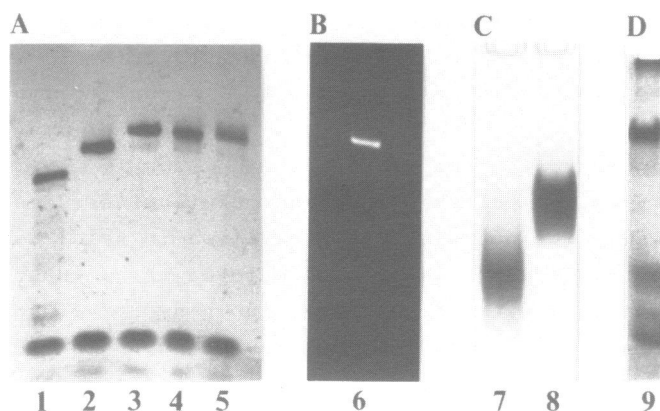


Fig. 3. PAGE analysis of labeled oligonucleotide hybridization probes. Panel A, UV shadowed 20% denaturing gel; panel B, long wavelength UV illumination of the panel A gel; panels C and D, UV shadowed 7% gels. 1, crude 20-base probe; 2, alkylamino derivative of the probe from 1; 3, biotin derivative; 4, isoluminol derivative; 5 and 6, fluorescein derivative; 7, AP probe; 8, AP. In 9, a crude reaction of HRP probe is shown. HRP is in the well, HRP-DNA is at the center of the gel, while unmodified DNA and dye are at the bottom. All fragments are 20 bases in length. In panel A, the lowest band in each lane is bromophenol blue.

analysis (enzyme labels), one synthesis can yield sufficient material for hundreds of thousands of assays.

To determine the detection limits of the various labeled probes in nucleic acid assays, we used a simple two-step sandwich method (29,30) as illustrated in Figure 2. The hybridizations were conducted in an identical manner for each probe using polystyrene beads or microtiter dish wells containing the capture probe. HRP, isoluminol and ^{32}P based assays were conducted on both solid phases. No significant difference was noted in the sensitivity (data not shown). Microtiter dish wells were preferred since the washing steps were considerably simpler to perform.

The assay detection limits (defined as the minimum quantity of analyte that gave a signal to noise ratio, S/N, of 2) are given in Table I. Also given are the detection limits for the labels and labeled probes. In Table II, representative results for sandwich assays performed in triplicate are presented.

Table I
Detection limit determinations.

Detection Limits- (in femtomoles)	Label Alone	Labeled Probe	Probe in Assay
<u>Label (detection system)</u>			
In Sandwich Assay:			
Fluorescein	20	100	500
Texas Red	20	30	100
Rhodamine	5	20	100
Isoluminol	1	30	100
^{32}P	0.05	0.05	0.05
AP (NPP)	5	5	5
AP (NBT/BCIP)	0.2	0.2	0.5
HRP (OPD)	0.1	0.1	0.1
HRP (LUM)	1	1	1
HRP (ENH/LUM)	0.05	0.05	0.05
In HBV assay:			
Fluorescein	-	-	100
HRP (ENH/LUM)	-	-	0.02

See Materials and Methods for abbreviations, assay and detection procedures.

Analyses were performed with and without the target fragment (absolute signal, S, and noise, N, respectively). The "no label" column shows the contribution of the reagents and/or instruments to backgrounds. The difference between N and the "no label" controls is an indication of the amount of NSB of the labeled probe.

Several observations can be made from Tables I and II. For fluorescent (fluorescein and rhodamine) and chemiluminescent (isoluminol) probes, a considerable loss in sensitivity is observed when comparing the labeled probes to the labels alone (Table I). Another significant decrease in sensitivity is realized in the hybridization assays (Table I). This is due not only to quenching upon hybridization, but also to NSB of the

Table II
Sandwich assay results.

Label (detection system)	S	N	S/N	no label	[target] ⁵
Fluorescein ¹	5822 ±493	2325 ±231	2.5 ±0.3	100 ± 50	500
Texas Red ¹	1649 ±183	782 ±138	2.1 ±0.4	100 ± 50	100
Rhodamine ¹	4422 ±347	2221 ±570	2.0 ±0.5	100 ± 50	100
Isoluminol ²	2.7 ± 0.4	1.2 ± 0.6	2.3 ±1.2	0.10 ±.01	100
AP (NPP) ³	0.60 ±.02	0.29 ±.01	2.1 ±0.1	0.26 ±.01	10
³² P ⁴	118 ± 18	36 ± 7	3.2 ±0.8	25 ± 6	0.05
HRP (OPD) ³	.014 ±.007	.003 ±.001	5 ± 3	.002 ±.001	0.10
HRP(ENH/LUM) ²	192 ± 7	32.7 ±7.3	5.9 ±1.3	12.7 ±0.5	0.10

See Materials and Methods for abbreviations. 1, in absolute fluorescent counts; 2, integral light output in relative luminescent counts; 3, absolute visible absorbance at maximum; 4, Cerenkov counts; 5, in femtomoles.

labeled probes (compare N and "no label", Table II). The NSB apparently arises from the hydrophobic nature of the labels, since, in each case, the fluorescent and chemiluminescent probes gave considerably higher backgrounds than a ³²P labeled probe employed at comparable concentrations. On a mole basis, fluorescent and chemiluminescent probes gave 4100-19,100 fold higher NSB than a probe of the same length and composition labeled with ³²P (Table II; $NSB \approx [target] \times [(N - no\ label) \div S]$; $NSB_{fluorescein} = 191\ fm$; $NSB_{Texas\ Red, rhodamine, isoluminol} = 41-48\ fm$; $NSB^{32P} = 0.01\ fm$).

On the other hand, binding either HRP or AP to a probe and subsequent hybridization had little effect on the activity of the labels, independent of the detection method. Also, NSB was less problematic. It is noteworthy that AP with NBT/BCIP and HRP with either OPD or ENH/LUM compare favorably in terms of detection limits with ³²P as a label. HRP was found to be a more sensitive label than AP by 10 fold (ENH/LUM versus NBT/BCIP, respectively). In addition, the time required for detection is considerably less for HRP (30 sec for ENH/LUM with HRP versus 18 h for NBT/BCIP with AP). For HRP analysis, the relative precision of the luminescent methods was far greater than that of the colorimetric detection system (Table II).

The addition of carrier substances (BSA, sonicated salmon sperm DNA, poly-A) and detergents (NP-40 and SDS) was found to significantly decrease NSB for all the labeled probes employed. Over coating of the polystyrene beads and plates with the same substances has also proved helpful. However, we were unable to fully eliminate analyte-independent binding. Probe length and composition are important criteria in decreasing label-independent NSB. Fragments of 18-20 bases with a G:C content of no more than 60% containing one label were found to be optimal in the sandwich assay method investigated here.

The simple assay described in Figure 2 was compared to the analysis of hepatitis B viral DNA with a solution phase sandwich method as described elsewhere (8) using fluorescein and HRP labels (Table I). Since six labeled probes were used per hepatitis genome (8), a considerable improvement in detection limit was realized while maintaining the relative efficiency of the enzyme versus fluorescent label.

DISCUSSION

N⁴-alkylamino deoxycytidine has been introduced into polynucleotides by nick translation with N⁴-(6-aminohexyl) dCTP (31), by bisulfite catalyzed transamination (32) and by the coupling of fully protected N⁴-alkylamino cytidine 3'-phosphoramidite during automated synthesis (9). There are significant advantages to the incorporation of the cytidine analog through chemical DNA synthesis. The modified nucleotide can be added in any number and at any position within the probe. Large scale synthesis of a variety of non-isotopically labeled derivatives can be conducted readily from commercially available or easily synthesized amine-specific labeling reagents. The use of small single strand synthetic probes offers the opportunity to purify the fully labeled oligonucleotide from partially and unmodified material by PAGE, providing an excellent resolution of all the reaction mixture components. The resulting probes can therefore be labeled at the maximum possible specific activity. Also, the deoxycytidine derivative can be synthesized on a large scale inexpensively and in good yield (57% overall; 8,33).

We did not anticipate a significant destabilization of

hybrids formed with oligonucleotides containing N⁴-alkylamino deoxycytidine since upon alkylation of exocyclic amines, K_{eq} for purine-pyrimidine association is not greatly diminished (34). Recently, the T_m determinations of N⁴-alkylamino deoxycytidine substituted versus unmodified polynucleotides have shown that duplex stability is essentially unaffected (B. Warner, unpublished results; 31,35).

It has often been noted in immunofluorescent procedures that "over labeling" can lead to non-specific staining (36). The use of fewer than 1 label in 20 nucleotides could perhaps decrease the NSB observed with fluorescent probes. The observed decrease in absolute fluorescence for oligonucleotide derivatives (Table I) is probably similar to that reported for fluorescent labeled antibodies where the presence of aromatic amino acids is known to cause quenching (37). The length and composition of the linker arm to nucleotides may be important, as has been noted for both biotinylated (2) and fluorescein labeled probes (7); however, the 10 atom spacer employed here is near the reported optimum. In the simple hybridization assay system utilized, photobleaching is an unlikely problem, although we did not investigate the addition of retardant compounds such as propyl gallate and p-phenylenediamine (38,39). Since a 10-fold decrease in NSB or quenching would not lead to an adequate detection limit, we did not pursue modification of the fluorescence systems. New instrumentation may greatly improve the detection limits observed with fluorescent probes (5).

The moiety to which isoluminol derivatives are bound can greatly affect the detection limit. For instance, the detection limit reported for the ABEI adduct of thyroxine is 20-100 times poorer than for ABEI alone (40). A similar decrease in sensitivity was observed for the oligonucleotide probe reported here (Table I; 30 fold decrease). The detection limit for isoluminol reported in Table I is consistent with previous reports (26,40).

In contrast to the fluorescent and chemiluminescent labels tested, little loss in sensitivity is noted upon attachment of enzymes to probes or as the result of hybridization of the labeled probes to the solid supported target fragment. Label to

label comparison (Table I) suggests that enzymes such as HRP and AP are the superior non-radioisotopic reporter groups and provide sensitivities nearly equivalent to ^{32}P . Although the detection limits of HRP with the various detection reagents are consistent with previous reports (27,28), our observed limit of detection for AP (enzyme alone and labeled probe) with NBT/BCIP is considerably poorer than reported elsewhere for AP probes used in dot blot formats (Table I, 500 attomoles; Ref 4, 2 attomoles; Ref 13, 24 attomoles). To some extent, the discrepancy could be due to the assay formats and solid phases employed.

Lysine residues are a particularly useful point of attachment of enzymes to oligonucleotides, given their usual abundance and the ease by which specific conjugation can be achieved. Due to the minimal perturbations on the enzymatic activities noted, it seems likely that the site of oligonucleotide incorporation on the HRP and AP labels is remote with respect to the active sites. Occasionally, amine modification can significantly alter enzyme activity; however, several alternative enzyme derivatization strategies can be employed (41).

The use of alternative fluorescent reporters (42) or chemiluminescent moieties (43) may alleviate the shortcomings of the labels used in our studies. Appropriate molecular design that takes into consideration electron density changes upon attachment, hydrophobicity and hydrophilicity, quenching and orientation may eventually lead to adequate small molecular labels. However, many of the best molecular reporting functions for direct labeling (eg, isoluminol) have also been tailored to serve as enzyme substrates (eg, luminol oxidation by HRP). This coupled with the intrinsic amplification provided by enzymes with high substrate turnovers significantly favors enzyme labeling methods. Using the HRP labeling strategy presented here and an amplification system based on chemically cross-linked oligonucleotides, we have been able to detect as little as 0.1 attomoles (60,000 molecules) of hepatitis B virus in human serum samples (9). Enzyme labeling schemes for oligonucleotides may eventually lead to detection limits of less than 100 molecules (44).

In conclusion, facile introduction of fluorescent, chemiluminescent and enzyme labels into synthetic oligonucleotide hybridization probes has been demonstrated using an N⁴-alkylamino deoxycytidine derivative. Although reports of the synthesis and use of fluorescent (5-7), chemiluminescent (45), and enzyme (4,8,9,13) labeled oligonucleotide hybridization probes have appeared elsewhere, to our knowledge no direct comparison of the detection limits achievable with these materials has been reported. We have shown that two enzymes commonly employed in immunoassays, HRP and AP, are superior to fluorescein, Texas Red, rhodamine and isoluminol as non-radioisotopic reporter functions in nucleic acid analysis based on the observed detection limits in a simple sandwich assay system.

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